2002

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Preparation of Clinical-Grade Recombinant Canarypox–Human Immunodeficiency Virus Vaccine–Loaded Human Dendritic Cells

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Preclinical data are reported that support a human immunodeficiency virus (HIV) vaccine strategy using recombinant canarypox–HIV vectors (ALVAC-HIV) to load human dendritic cells (DCs) with HIV antigens. Clinical-grade DCs were infected with good manufacturing practice–grade ALVAC-HIV vaccine constructs. ALVAC infection, HIV gene expression, and DC viability and function were monitored by use of immunohistochemistry, flow cytometry, blastogenesis assays, antigen-specific interferon (IFN)–γ enzyme-linked immunospot assay, and enzyme-linked immunosorbent assay protein detection. The vaccines infected both immature and mature DCs, and intracellular HIV-1 Gag protein was detected within hours. ALVAC-HIV induced DC maturation that was mediated by tumor necrosis factor–α and induced DC apoptosis that was directly related to the length of vaccine exposure. Of importance, the infected DCs remained functional in T cell stimulation assays and induced HIV antigen–specific $CD8^+$ T cell production of IFN-γ from cells of HIV-1–infected individuals. These data support an ongoing HIV vaccine trial comparing conventional vaccine delivery routes with ex vivo vaccine–loaded autologous DCs for immunogenicity in HIV-1–uninfected volunteers.

Live viral vectors are being used increasingly as an effective means of delivering vaccine immunogens. Recombinant canarypox (ALVAC)–human immunodeficiency virus (HIV) type 1 vaccines have been administered with a good safety profile to >2000 individuals, mainly among HIV-seronegative populations [1–7]. However, these vaccines are only modestly immunogenic, as defined by the elicitation of antigen-specific cytotoxic T cells, in about one-third of volunteers [8, 9]. The generation of T cell immunity requires dendritic cells (DCs), which convert antigens into immunogens [10]. This role gives rise to their description as "nature's adjuvant" and is one reason why new immunization and immunity-monitoring strategies often incorporate DCs [11, 12]. Recent advances in techniques for the cytokine-induced differentiation of monocytes into DCs [13, 14] have provided easier access to this important antigen-presenting cell (APC), which had been a scarce cell type since its discovery 30 years ago [15, 16]. Many clinical studies using autologous DCs loaded with tumor antigens (immunotherapy) are in progress [17–22]. These DCs induce antitumor immunity, such as expanded cytotoxic T cell (CTL) activity within the $CD8^+$ compartment [17]. In healthy human volunteers, a single injection of mature monocyte-derived human DCs loaded with multiple antigens (i.e., tetanus toxoid [TT], influenza matrix peptide, and keyhole limpet hemocyanin) expands both $CD4^+$- and $CD8^+$-specific immune responses [23]. The level of maturation of the DCs may be of critical importance in the nature of the immune response to DC-delivered antigen [24–26]. Recent studies suggest that antigen-loaded mature DCs induce a protective (T helper type 1) immune response, whereas antigen-loaded immature DCs may "silence" the immune response via expansion of interleukin (IL)–10–producing T cells.

With the hope of engineering better vaccines, new strategies are being used in an attempt to target DCs. Since we planned to test the in vivo immunogenicity of canarypox (CP) vectors delivered via DCs, we report our preclinical work on the ex vivo

Received 13 March 2002; revised 12 June 2002; electronically published 7 October 2002.

Presented in part: 8th Conference on Retroviruses and Opportunistic Infections, Chicago, 4–8 February 2001 (abstract C34e).

Informed consent was obtained from all volunteers. The research was conducted within the ethical guidelines of the authors' institutions and within human experimentation guidelines of the US Department of Health and Human Services. Clinical trial RV100 (source of frozen aliquots of peripheral blood mononuclear cells used to generate dendritic cells) was institutional review board–approved and followed all relevant federal guidelines and institutional policies.

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The Journal of Infectious Diseases 2002; 185:1242–52

0022-1899/2002/18509-0006$15.00

infection of immature and mature human DCs with existing ALVAC-HIV vaccine candidates. We describe the reproducible generation of large numbers of clinical-grade human DCs and the interactions of these cells with good manufacturing practice (GMP)–grade ALVAC-HIV vaccines. The early expression of the HIV p24 *gag* gene in transfected DCs was demonstrated by fluorescence-activated cell sorter analysis and immunohistochemistry. In addition, these studies show maturational effects of the vector on DCs, with preservation of APC integrity and function in vitro.

Materials and Methods

Generation of clinical-grade human DCs. We adapted published methods [27], using leukapheresis (RH Laboratories) as a source of monocyte precursors to generate DCs. In brief, leukapheresis blood products (ACD-A anticoagulation) were diluted 1:1 in PBS or RPMI 1640 medium (BioWhittaker) and layered over Ficoll-hypaque (Amersham Pharmacia Biotech) in a blood-to-ficoll ratio of 2:1, to isolate peripheral blood mononuclear cells (PBMC). The PBMC were washed repeatedly with PBS or RPMI 1640 at successively lower *g* forces (250, 170, and 110 *g*) to remove platelets. The PBMC then were frozen in X-VIVO 15 medium (BioWhittaker) supplemented with 12.5% human serum albumin (Alpha Therapeutics) and 10% dimethyl sulfoxide (Sigma). They were initially frozen at −80°C overnight and then transferred to the vapor phase of liquid nitrogen the next morning. Before use, the cells were rapidly thawed at 37°C, and complete medium (cRPMI) was added dropwise to the suspension. cRPMI is RPMI 1640 supplemented with 1% autologous human plasma (heat inactivated), 2 m*M* L-glutamine (BioWhittaker), and 20 mg/mL gentamicin (American Pharmaceutical Partners). The cells were gently pelleted (100 *g*) and resuspended in cRPMI at 5×10^6 cells/mL, and 10 mL were plated onto Falcon cell culture dishes (BD Biosciences).

The thawed, cryopreserved cells were cultured for 1 h at 37°C in 5% CO_2, and then the nonadherent cells were rinsed off. Fresh cRPMI (10 mL) was added, and the adherent cells were placed into culture overnight. The following day, the medium was replaced with fresh cRPMI. Recombinant human IL-4 (800 U/mL; Cell Genix) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1000 U/mL; Immunex) were added on days 1 and 3. On day 5, the cells were replated into 6-well plates at 5×10^5 cells/well in 3 mL, and the cytokines (IL-4 and GM-CSF) were added again. To generate mature DCs, 0.75 mL of autologous monocyte–conditioned medium (MCM) was added to each well (20% vol/vol) on day 6. MCM contains the cytokines needed to mature DCs (i.e., IL-1β, IL-6, tumor necrosis factor [TNF]–α, and prostaglandin-E_2) [27, 28]. On day 7, the cells were ready for baseline phenotyping (flow cytometry) and loading with CP either as immature (without MCM) or mature (with MCM) DCs.

The phenotype of the DCs was routinely monitored by 2-color fluorescence-activated cell sorter analysis on a FACScan flow cytometer (Becton Dickinson) using BDIS/PharMingen phycoerythrin (PE)–conjugated monoclonal antibodies to IgG_1, IgG_{2b}, CD1a, CD3, CD14, CD20, CD25, CD80, CD86, and HLA-DR. PE-conjugated monoclonal antibodies to CD40 and CD83 were from Immunotech Beckman Coulter.

MCM preparation. Frozen PBMC (and, in some experiments, fresh PBMC) were used to generate MCM. Falcon cell culture dishes used to generate MCM were pretreated with human intravenous immunoglobulin (IVIG; gift from Bayer, North America) in PBS (100 μg/mL) for 10 min at room temperature. The plates were washed 3 times with 10 mL of PBS to remove excess IVIG. Cells were thawed and plated onto IVIG-coated plates at 5×10^6 cells/mL in 10 mL, as described elsewhere [27]. The plates were incubated overnight, and the cell-free MCM supernatant was collected, filtered (0.2 μL), and frozen at −80°C prior to use.

ALVAC HIV vaccine constructs. All ALVAC-HIV vaccines were obtained from Aventis Pasteur. CP viruses are members of the avipox genus of the *Orthopoxvirus* family of DNA viruses and are highly restricted in terms of productive replication to avian species. ALVAC-HIV (vCP205) is a preparation of recombinant CP virus expressing the products of the HIV-1 *env, gag,* and *pro* genes. These genes are inserted into the C3 locus and are regulated by the vaccinia virus H6 and I3L promoters. vCP1433 is equivalent to vCP205 with the addition of *nef* and *pol* CTL epitopes. vCP1452 is equivalent to vCP1433 under different promoters (E3L and K3L) and vCP1521 is *env*/*gag*-clade E/*pro* (similar to vCP205).

Loading DCs with CP. Preliminary work was done using laboratory-grade CP constructs that were suspended in serum-containing media solutions, as described elsewhere [29]. When transitioning to using the GMP-grade lyophilized vaccines, there was a rapid loss of cell viability after exposure to vaccines that were resuspended as directed for routine injection (1 mL of sterile H_20). However, when the vaccines were resuspended in enough sterile H_20 (range, 1.6–1.9 mL) to achieve physiologic osmolality (280–300 milliOsmoles [mOsm]), the DCs remained viable. The vaccines ranged in titer from $10^{6.4}$ to $10^{6.8}$ $TCID_{50}$, as determined by plaque assay. A single vial of vaccine ($\sim 10^{6.5}$ infectious virus particles) was used to load $2–3 \times 10^6$ DCs, which resulted in an MOI of 2.5. The cells were exposed to the vaccine for 2 h (or less in some experiments) at 37°C in 5% CO_2. The cells were washed and counted, and viability was checked by use of trypan blue exclusion and, in some cases, annexin-V and propidium iodide (PI).

Determination of infection rates and gene expression. After vaccine exposure, the cells were washed, resuspended in cRPMI at 10^6 cells/mL, and placed back into culture. At specified time points after vaccine exposure, the cells were washed, fixed, permeabilized (PharMingen Fix and Perm Kit), and stained intracellularly for both CP (polyclonal rabbit anti-CP virus serum at 1:10,000 dilution; Virogenetics) and HIV-1 p24 (murine monoclonal antibodies at 1:10 dilution; Dako) antigens. These primary antiviral antibodies were detected by use of PE-conjugated secondary antibodies (goat anti-rabbit or goat anti-mouse) at 1:200 dilutions (Southern Biotechnologies Associates). In some experiments, the cells were double labeled using anti-p24, as described above, and anti–CD83–fluorescein isothiocyanate (FITC)–conjugated monoclonal antibodies.

Maturation (TNF) blocking studies. DCs were exposed to vaccine or medium, usually for 2 h, and then were washed with fresh cRPMI to remove excess virus. The exposed cells were split into 2 aliquots and were either treated with IgG_1 control antibody (PharMingen) or neutralizing anti–TNF-α antibody (R&D Systems) at a concentration of 20 μg/mL, as described elsewhere [29]. The cells were collected within 24 h, and surface staining for maturation markers was performed.

Apoptosis assay. In some experiments, annexin-V and PI stain-

ing kits (PharMingen) were used according to the manufacturer's guidelines to identify cells undergoing apoptotic and/or necrotic cell death.

Immunohistochemistry. After vaccine exposure, ~2×10^5 DCs were spun onto glass slides, using a Cytospin (Shandon Lipshaw) set at 50 *g* for 10 min. After being air-dried, the slides were fixed in acetone and stored at −20°C until staining commenced. The slides were warmed to room temperature, rehydrated in PBS, and permeabilized with 0.1% saponin (Sigma) for labeling of intracellular antigens. All antibodies were diluted in the appropriate "blocking" serum and incubated for 30 min with antibodies at room temperature. Slides were washed for 10 min in PBS with gentle stirring. Staining was done in parallel on unexposed cells (mock infected), and appropriate isotype controls were used to assess background for each experiment.

Lymphoproliferation assays. Allogeneic T cell proliferation assays were performed as follows. T cells were isolated from PBMC by negative selection, using a T cell–negative isolation kit (Dynal Biotech) containing antibodies to CD14, CD16, CD56, and HLA-DR/DP, according to the manufacturer's guidelines. In brief, the cells were incubated with the antibody cocktail for 10 min at 4°C and then washed to remove excess antibody. Next, the magnetic beads were added, and the cells were gently rotated at room temperature for 15 min. The cell suspension was placed into a magnetic field to remove unwanted cells (those other than T cells). To enhance purity, the T cells were placed into the magnetic field twice. These T cells were plated into 96-well round-bottom plates (Costar) in triplicate and cocultured at 2×10^5 cells/well with various concentrations of DCs. Alloreactions were incubated at 37°C under 5% CO_2 for 3–5 days, pulsed with 1 μCi/well [^{3}H]thymidine (DuPont) for 18 h, and harvested onto filter mats for scintillation counting in a 1450 Microbeta Trilux (EG&E Wallac).

Autologous T cell proliferation assays were done as follows. These assays were performed in the same manner as the above alloreactions, with the exception that autologous experiments used 1×10^5 responder cells and were incubated for 5–6 days. TT (3.2 limits of flocculation) was added directly to the wells. After incubation, the cells were pulsed with 1.6 μCi/well [^{3}H]thymidine for 18 h, harvested, and counted as described above. Supernatants (100 mL/well) were collected immediately prior to the thymidine pulse and frozen (−80°C) until analyzed for interferon (IFN)–γ production.

Antigen-specific IFN-γ production detection using ELISPOT assays. DCs were generated from frozen aliquots of PBMC from HIV-seropositive individuals who had undergone leukapheresis as part of a clinical trial (RV100). The cells were thawed and prepared as described above for seronegative donors. In general, the ELISPOT assays were performed by directly stimulating PBMC with the antigens or by stimulating PBMC with antigen-loaded DCs. The PBMC were added to the assay plates at 2×10^5 cells/well. Ninety-six–well ELISPOT plates (Multiscreen-IP MAIP type plates; Millipore) were prepared by precoating with mouse anti–human IFN-γ antibody (MAb 1-D1K; Mabtech) at 5 μg/mL in 50 mL of PBS overnight at 4°C. Plates were washed 5 times with PBS and blocked with 100 mL of complete medium (RPMI 1640 medium [BioWhittaker] and 10% normal human serum [Sigma]) for 1 h at 37°C.

For evaluation of CD8 T cell dependence, the PBMC were split into 2 aliquots and treated with Dynabeads M-450 CD8 or M-450 sheep anti–mouse IgG (Dynal Biotech) for CD8 and sham depletions, respectively. The immunomagnetic beads and cells that adhered to the beads were removed with a magnet, and the resulting cell populations were washed twice and resuspended in complete medium. On the basis of the cell count in the sham depletion, between 1×10^5 and 2×10^5 PBMC were added to each well of the precoated and blocked ELISPOT plate. vCP205- or vCP1452-exposed DCs (or mock or ALVAC empty vector infected) were washed once after infection and distributed in duplicate or triplicate wells at a count of 0.5×10^4–1×10^4 cells/well (ratio of 20:1, PBMC:DCs). The negative control and ALVAC control wells were all done in quadruplicate. As a positive control for functional integrity of the cells, either phytohemagglutinin-P or staphylococcal enterotoxin–B was added to duplicate wells at a final concentration of 5 μg/mL. For the direct vCP205 addition experiments, 2×10^5 PBMC were added to each well of the ELISPOT plates, and ALVAC was added directly to the wells at 5 pfu/cell. Plates were incubated for 20–24 h at 37°C (5% CO_2) and were then washed with PBS/0.05% Tween 20 buffer and incubated for 2 h with mouse anti–human IFN-γ antibody conjugated with biotin (7B6-1–biotin; Mabtech). Development consisted of a 1-h incubation with an avidin–horseradish peroxidase complex (Vectastain ABC kit; Vector Laboratories), followed by washing (PBS/0.05% Tween 20 buffer) and incubation with peroxidase substrate AEC (Vector Laboratories) for 4 min.

ELISA-based cytokine detection assays. TNF-α was detected as a secreted protein product in culture supernatants by use of a specific commercial ELISA assay (R&D Systems). Assays were performed according to manufacturer's guidelines. The lower detection limit for TNF-α was 16 pg/mL.

Statistical analysis. Microsoft Excel 98 was used for statistical analysis of data (paired Student's *t* test).

Results

CP infection and HIV Gag protein expression in GMP vaccine–exposed DCs. DCs were generated from frozen aliquots of PBMC from donors who underwent leukapheresis, and, if indicated, matured with MCM, as described elsewhere [27]. On average, we obtained a 5.3% ± 0.3% yield ($n = 42 \pm$ SEM) of fully mature DCs from each culture. Figure 1*A* shows the typical phenotype of immature and mature (after MCM exposure) DCs. In particular, mature DCs express high levels of CD83 and CD86. There is minimal contamination from T and B cells. Figure 1*B* shows the morphology of mature DCs in culture on day 7.

Immature and mature DCs were exposed to ALVAC-HIV and evaluated for expression of CP and HIV antigens. For intramuscular use (the usual delivery route), the lyophilized vaccine is resuspended in 1 mL of H_20. However, this results in supraphysiologic osmolality, which is not tolerated by DCs in culture. Thus, prior to exposure of DCs to ALVAC-HIV, we adjusted the volume of the vaccine to 1.6–1.9 mL to provide physiologic osmolality (i.e., 280–300 mOsm). After vaccine exposure, the DCs were analyzed at both early (4–6 h) and late (18–24 h) time points to determine infection rates (CP) and HIV Gag protein expression (p24), using a polyclonal anti-CP

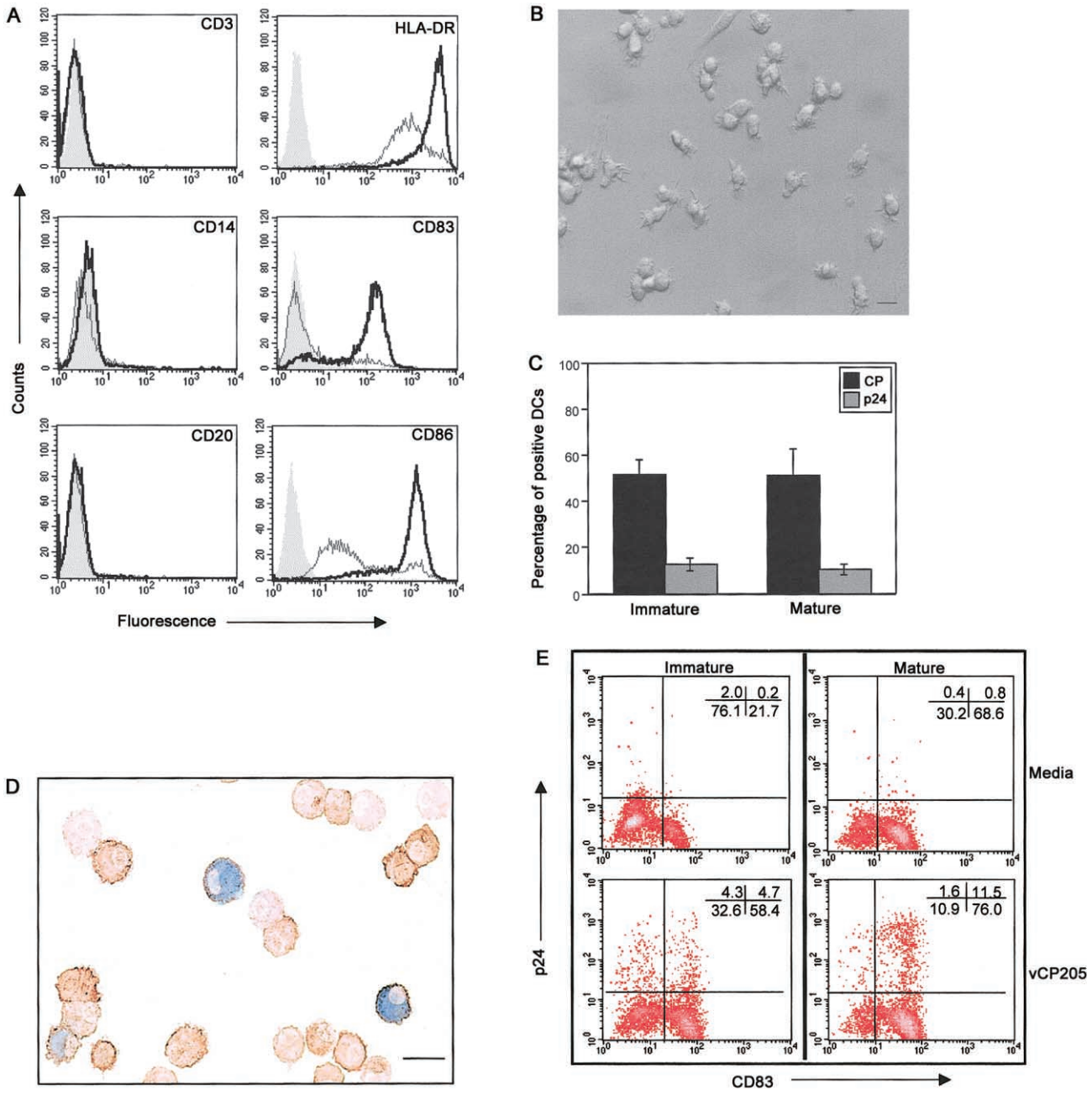

Figure 1. Dendritic cell (DC) phenotype, DC morphology in culture, and canarypox (CP) and p24 *gag* expression in DCs. *A,* DCs prepared by adherence and matured *(heavy black line)* using monocyte-conditioned media (MCM) contain infrequent contaminating T (CD3) or B (CD20) cells and express minimal CD14 and high levels of HLA-DR, CD83, and CD86. Immature DCs (no MCM; *fine black line*) demonstrate lower levels of HLA-DR, CD83, and CD86. Filled gray histogram represents the relevant isotype controls. *B,* Phase contrast microscopy demonstrating nonadherent mature DCs in a 6-well culture dish on day 7 (scale bar, 20 μm). *C,* Immature and MCM-matured DCs were exposed to vaccine for 2 h, permeabilized, and then labeled with anti-CP and p24 antibodies for fluorescence-activated cell sorter analysis, as described in Materials and Methods. The histograms represent mean CP and p24 expression ($\pm$SEM) 4 h after vaccine exposure ($n = 3$ donors). *D,* Immunohistochemistry staining for intracellular p24 *(blue)* and HLA-DR *(brown)* at the 4-h time point shows that all cells are HLA-DR$^+$ and that ~15% are expressing intracytosolic p24 (scale bar, 20 mm). *E,* Distribution of expressed intracellular p24 protein (within immature or mature DCs populations) depends on initial maturity levels of the exposed DCs. There is equal distribution of p24 in CD83$^+$ and CD83$^-$ fractions when the majority of starting DCs are immature *(left)*. If the DCs are mature when exposed *(right)*, most p24 protein colocalizes within the CD83$^+$ DCs. A representative experiment is shown, conducted 18 h after vaccine exposure ($n = 3$).

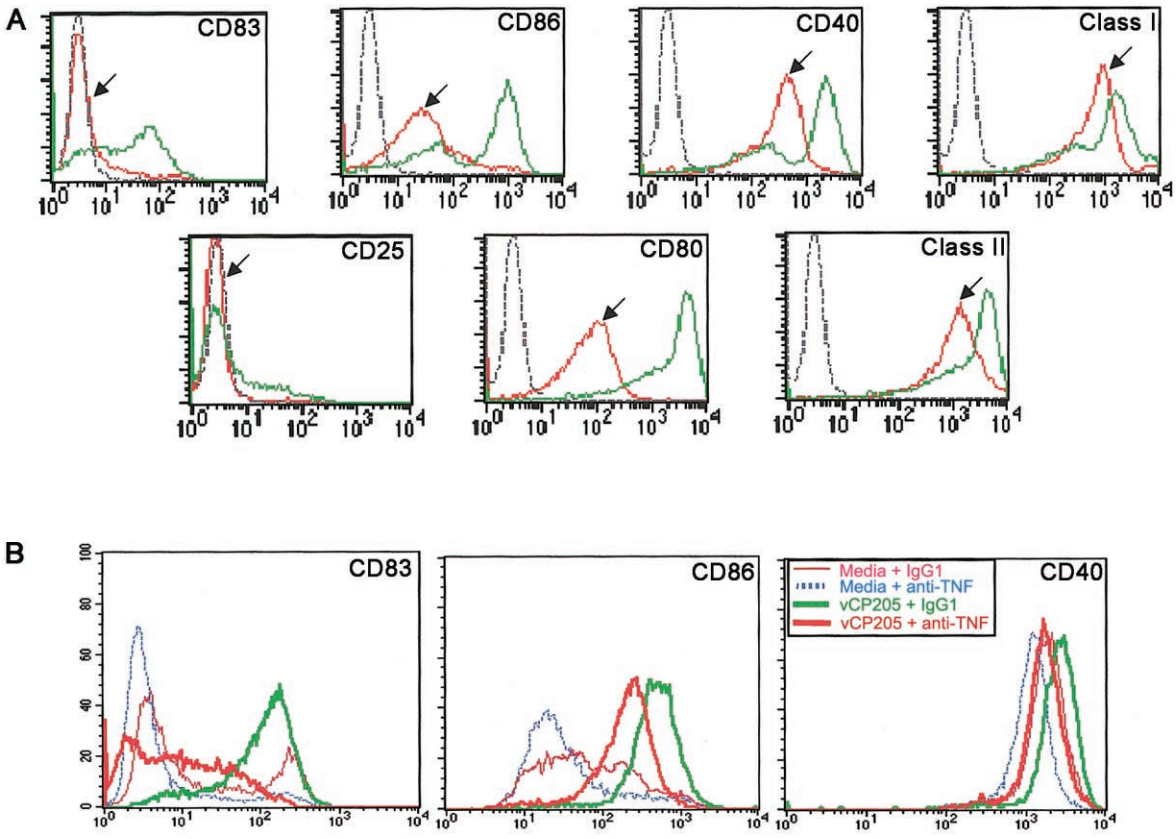

Figure 2. Maturation induced by exposure to canarypox vaccine. *A,* Immature dendritic cells *(arrow)* without vaccine exposure and subsequent phenotype observed 24 h after vaccine exposure demonstrate profound maturation with increased expression of all markers in a representative donor ($n = 5$). *B,* Use of a neutralizing anti–tumor necrosis factor (TNF)–α antibody can partially block vaccine-induced maturation, as shown by the inhibition of CD83, CD86, and CD40 up-regulation in this representative blocking experiment ($n = 3$).

antibody preparation and a monoclonal anti-Gag antibody. The percentage of cells positive for CP or Gag antigens was determined by flow cytometry, using relevant isotype controls and mock-infection conditions to set gates for background staining. Figure 1*C* shows that exposure of immature or mature DCs to ALVAC-HIV results in expression of CP antigens in ~50% of DCs. About one-quarter of the CP-positive DCs also expressed HIV-1 Gag protein. Figure 1*D* shows the immunohistochemical staining of intracellular p24 protein (blue) distributed throughout the cytoplasm of ~15% of the DCs at 4 h after ALVAC-HIV infection. The brown stain labels surface HLA-DR and identifies these cells as major histocompatibiltiy complex (MHC) class II–bearing APCs. Figure 1*E* shows flow cytometric dot plots of DCs stained for surface CD83 and for intracellular p24 Gag (note the 18-h time point in the figure legend). When infecting initially immature DCs, note that the p24 protein was evenly distributed between immature and resultant mature DC populations *(lower left);* however, when starting with a more mature population *(right panel),* most p24 protein was colocalized to mature $CD83^+$ DCs *(lower right).*

Vaccine-induced maturation of clinical-grade cultured human DCs. As we reported elsewhere [28], using a laboratory-grade vector, CP infection of DCs results in a TNF-α–mediated maturational effect on immature DCs. Using the GMP-grade ALVAC-HIV vaccine, we also noted a profound maturational effect (figure 2*A*), which was mediated in part by TNF-α (figure 2*B*). Nonrecombinant ALVAC (empty vector) also induced maturation of DCs (data not shown). In most donors, full maturation of DCs was observed within 24 h of CP infection. The arrows in figure 2*A* represent the immature DC phenotype under mock-infection conditions (medium only). This represents the starting population phenotype. After vaccine exposure and overnight incubation, most of the cells matured, as evidenced by the increased expression of all surface markers shown (figure 2*B*). However, only the expression of CD83, CD86, and CD40, as measured by mean fluorescence intensity, were consistently and significantly inhibited (>50%) in the presence of the anti-TNF antibody. In the blocking experiments, we used an anti–TNF-α antibody that blocked both soluble and membrane-bound TNF-α or an IgG control antibody in both mock- and vaccine-infected DCs. The vaccine-induced maturation effect can be appreciated in figure 1*E,* with an overall

increase in CD83 expression in both DC populations after vCP205 exposure (figure 1*E, left panel, top to bottom,* CD83 increased from 22% to 63%; *right panel, top to bottom,* CD83 increased from 69% to 88%). Of interest, we observed that immature DCs produced ~10-fold more TNF-α than did mature DCs (571.4 ± 128 vs. 61.0 ± 30.5 pg/mL; $n = 5$ donors; $P < .009$) after vaccine exposure. Since the mature DCs were readily infected with CP, we focused on loading mature cells.

Similar infection rates and Gag expression using various vaccine constructs. Several CP HIV-1 vaccine constructs have been developed for clinical studies. Most human clinical trial experience is with an earlier construct, vCP205. Subsequent constructs included additional HIV-1 genes and different promoter control. We compared the rates of expression of CP antigens and HIV Gag protein 4–6 h after exposure to 4 different ALVAC-HIV vaccine constructs and 2 different lots of vCP205. As shown in figure 3*A,* all ALVAC-HIV constructs displayed similar levels of CP and p24 Gag expression in DCs. Of note, CP and Gag expression was measured at 4–6 h because this time point was optimal for vCP205 (maximal p24 Gag expression). In addition, we compared the 2 different lots vCP205 in titration assays using mature DCs as the target cells. The reported $TCID_{50}$ per vial of vaccine (range, $10^{6.4}$–$10^{6.8}$) was used to calculate the MOI. As can be seen in figure 3*B,* there was comparable CP infection and gag expression between the two vaccine lots of vCP205, with an optimal MOI of 2–5.

CP induction of apoptosis in vaccine-exposed DCs. An appealing feature of CP as a viral vector is that the virus is nonpathogenic for humans. However, several reports document that CP induces apoptosis in infected DCs [29, 30], with immature DCs being more susceptible than mature DCs. It has also been reported that CP-infected apoptotic DCs can be taken up by uninfected DCs and cross-presented for T cell stimulation in vitro [30]. Indeed, we found more cell death in immature DCs than in mature DCs after vaccine exposure. At 24 h after infection, the mean (± SEM) viability of immature and mature DCs was 64% (± 6.6%) and 80% (± 5.1%), respectively ($P <$ .018). Subsequent experiments focused on mature DCs to optimize susceptibility to CP infection while controlling for the amount of cell death. Using a sensitive assay involving a combination of annexin-V and PI staining, we detected more nonviable DCs (24 h after a 2-h vaccine exposure) than by using trypan blue exclusion alone (figure 4*A*). As expected, the trypan blue and PI measurements were closely correlated ($r = 0.8$), because annexin-V–labeled cells can still exclude trypan blue and PI. We also noted that after vaccine exposure, DC viability was influenced by the type of culture medium (RPMI 1640, PBS, or saline) used to maintain the cells after loading (figure 4*B*). The DC viability decreased substantially over time when maintained in PBS or saline, compared with RPMI 1640 medium. The DCs were much less affected by storage temperature (figure 4*C*), although 37°C seemed to be optimal.

Finally, we observed that the amount of induced apoptosis was directly related to the length of time the cells were exposed to the vaccine (figure 4*D*). Thus, with regard to ex vivo loading and delivery of DCs, the 2-h vaccine exposure would allow sufficient time for infection of DCs, with some downstream apoptosis, and for a largely viable product to be delivered.

Vaccine-exposed DCs retain APC function. To ensure that the DCs would remain functional despite being infected with a live viral vector, we used stimulation of the mixed lymphocyte reaction to assess DC function. Negatively selected responder T cells were cultured with titrated amounts of allogeneic DCs (exposed to vCP205 or saline). After 3–5 days of culture, the vCP205-DCs retained full and strong stimulatory reactivity, as shown in figure 5*A*. To test presentation of protein antigen by ALVAC-HIV–infected DCs, we studied autologous tetanus antigen-specific lymphoproliferation. After DC exposure to vCP205 vaccine or saline, DCs were added in graded doses to a fixed number of autologous responder T cells and then incubated with TT. After 5–6 days of culture, the cells were pulsed with [^{3}H]thymidine overnight and harvested as described above. Figure 5*B*

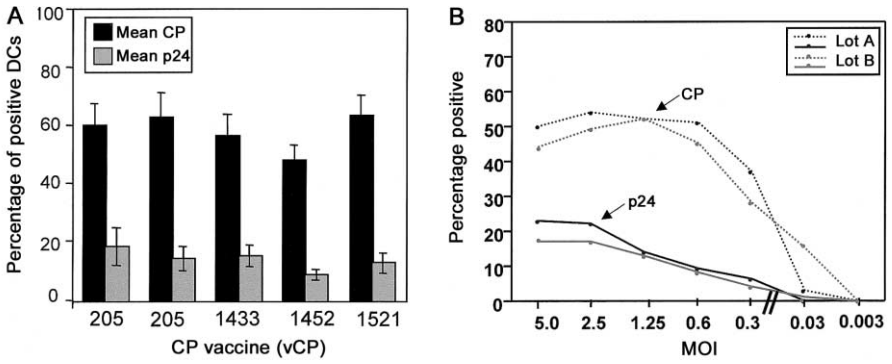

Figure 3. Similar expression of canarypox (CP) and p24 in mature dendritic cells (DCs), using various good manufacturing practices–grade lyophilized vaccine constructs (vCP205, vCP1433, vCP1452, and vCP1521). *A,* Histograms represent the mean CP and p24 expression (± SEM) 4 h after vaccine exposure ($n = 3$ donors). *B,* Vaccine titration curve in a representative donor (1 of 3 tested), using CP p24 antibody detection in fluorescence-activated cell sorter analysis comparing 2 different lots of vCP205 demonstrating an optimal MOI of 2–5.

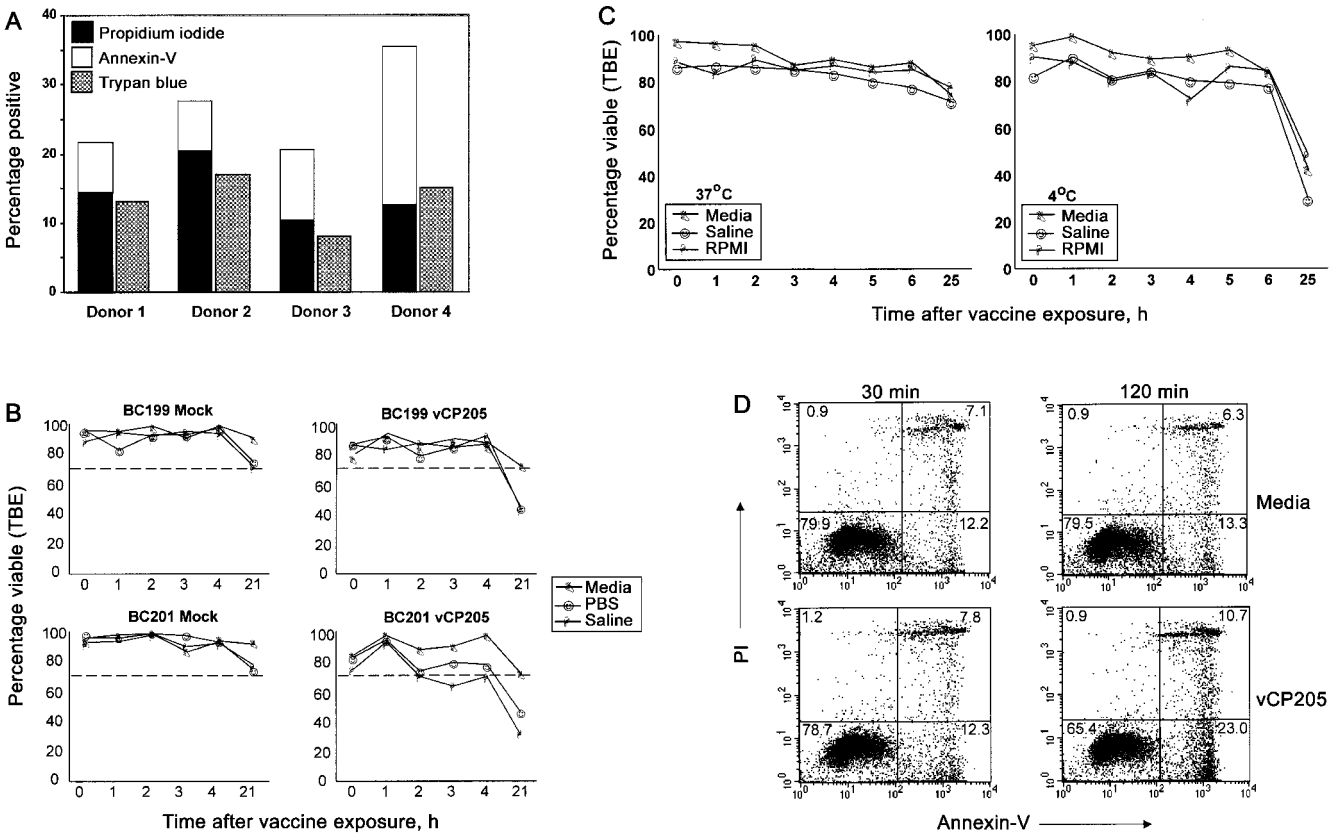

Figure 4. Canarypox-induced apoptosis in exposed mature dendritic cells (DCs). *A,* DCs were exposed to vCP205 for 2 h, and cell viability was assessed 24 h later by use of trypan blue exclusion (TBE) and an annexin-V propidium iodide (PI) assay. Annexin-V and PI are shown in darker stacked histograms, and the trypan blue–positive cells are shown in gray for individual donors ($n = 4$). *B,* DCs generated from frozen peripheral blood mononuclear cells from 2 different donors (thawed after 6–9 months of cryopreservation) were exposed to vCP205. Viability was monitored using TBE in cells that were resuspended in RPMI 1640 medium, PBS, or saline. *Dotted line,* 70% viability cutoff (required for release). Unexposed DCs (mock) remained more viable over time, and loaded DCs preferred medium to other solutions. Two of 3 representative experiments are shown. *C,* Temperature dependence of viability (TBE) of vaccine-loaded DCs from a representative donor (1/3) compared at 37°C vs. 4°C. *D,* Apoptosis measured 24 h after vaccine exposure (30 vs. 120 min) in a representative donor. Unexposed DCs kept in medium show 12%–13% annexin-V positivity. Similar levels of apoptosis were observed after brief exposure to the vaccine (30 min), but apoptosis nearly doubled with longer vaccine exposure times (120 min).

shows that vCP205-exposed DCs were more efficient than unexposed DCs in presentation of TT to autologous T cells. Finally, we tested the ability of vCP205-exposed DCs to present HIV antigens to sensitized T cells. We used the ELISPOT assay in an autologous system to detect antigen-specific $CD8^+$ T cell production of IFN-γ. Frozen PBMC were used to generate autologous DCs that were loaded with either the empty vector (ALVAC) or ALVAC-HIV (vCP205 or vCP1452). PBMC were added to the ELISPOT plate and either stimulated directly with medium (ALVAC, vCP205, or vCP1452) and phytohemagglutinin-A or stimulated via antigen-loaded DCs. vCP205-loaded DCs clearly induced HIV antigen–specific IFN-γ, which is largely derived from CD8, as demonstrated using CD4 and CD8 cell–depletion techniques (figure 5*C*).

Discussion

Live recombinant CP viral vectors represent a safe method for delivering vaccine antigens; however, immunogenicity may not be optimal under standard immunization conditions of intramuscular delivery. One approach to improve vaccine immunogenicity is to target the vaccine antigens directly to DCs in vivo. Here, we have studied the interaction of ALVAC-HIV constructs with monocyte-derived human DCs. We report preclinical findings, using an ex vivo vaccine–loading method to ensure that the DCs were infected with the vector and could express and present HIV-specific antigens.

We used GMP-grade lyophilized vaccine (vCP205 and other ALVAC HIV constructs) to infect clinical-grade human DCs. Both immature and mature DCs could be readily infected with

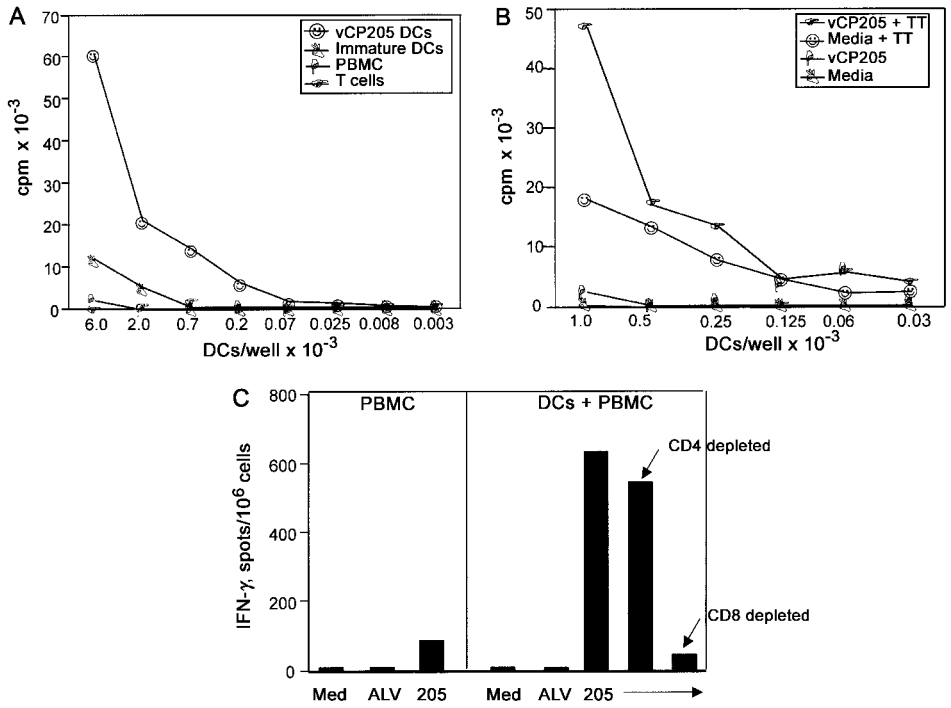

Figure 5. Retention of dendritic cell (DC) function after canarypox (CP) exposure. *A,* vCP205-exposed immature DCs induced higher levels of DNA synthesis, compared with unexposed immature DCs, in a standard alloreaction. *B,* In an antigen-specific autologous system, CP-exposed monocyte-conditioned medium (Med)–matured DCs induced a stronger recall proliferative response to tetanus, compared with unexposed mature DCs. *C,* In human immunodeficiency virus (HIV)–seropositive persons, using an interferon (IFN)–γ ELISPOT assay system, vCP205 (205)–loaded DCs but not recombinant CP (ALVAC)–infected DCs (ALV; empty vector) presented relevant HIV antigens, driving a CD8-specific response. Results are from a representative donor, with a minimum of 3 experiments performed. PBMC, peripheral blood mononuclear cells.

the CP vaccine; ~50%–60% of DCs expressed CP antigens and about one-third of these DCs expressed HIV Gag protein. In an effort to standardize DC immunization in planned vaccination trials, we used leukopheresis-derived mononuclear cells as the DC precursor source and MCM as the maturational signal [27]. Although there was some variability in maturation induced by MCM, we consistently found that >50% of the DCs expressed CD83 after exposure to MCM. Even with MCM-matured DCs, we observed further maturation of the cell population after exposure to vaccine. We infected immature and mature DCs and compared subsequent p24 expression patterns in both cell types. There was comparable CP infection and p24 Gag expression in both the initially immature and mature DC populations. However, the maturity of p24 Gag–expressing cells was different in the 2 cell types. Most p24 Gag–expressing cells were $CD83^+$ if the starting population was mature, but p24 was evenly distributed between $CD83^-$ and $CD83^+$ DCs if the starting population was immature. Although CD83 function is incompletely understood, it is commonly used as a marker of DC maturation, and its expression recently has been linked with the ability to induce protective (T helper type 1) immune responses to test antigens in human clinical trials [31]. Since it is important to have a population of mature DCs to elicit optimal immune responses, these data suggested that our planned clinical study should be done by loading mature DCs with CP. This approach minimizes the chance of injecting Gag-expressing immature DCs.

Exposure of immature DCs to ALVAC constructs resulted in a profound maturational effect that was partly mediated by TNF-α. This maturational effect with laboratory-grade CP vectors was reported elsewhere [29]. In contrast, vaccinia virus actually inhibits DC maturation [32] and function [33]. Similar inhibitory effects are seen with herpes simplex vectors [34, 35], whereas other viral vectors (e.g., adenovirus) do not influence maturation [36]. Regarding TNF-α production, the expression of this cytokine by skin DCs may be a beneficial feature of a vector, since TNF-α has been directly associated with the migration of Langerhans' cells out of the epidermis [37, 38]. In addition, the maturation state and the migratory ability of DCs are thought to be closely linked [39], although there are likely other factors influencing DC trafficking (e.g., chemokines) [40–42]. We found that immature DCs produced ~10-fold more TNF-α after vaccine exposure, compared with mature DCs. In addition, there was an increase in costimulatory markers, such

as CD25, CD40, CD80, CD86, and MHC classes I and II. The increased expression of some of these molecules (CD40, CD83, and CD86) could be substantially blocked by use of an anti–TNF-α blocking antibody, suggesting a role for TNF-mediated maturation. The increased expression of both types of MHC molecules after exposure to a virus is interesting and encouraging with regard to retention of APC function and stimulation of potential CD4 and CD8 responses.

A single injection of monocyte-derived mature DCs pulsed with keyhole limpet hemocyanin, influenza matrix peptide, and TT has been shown to expand specific CD4 and CD8 immune responses in healthy volunteers [23]. Recent reports indicate that the delivery of antigen-loaded immature DCs into humans can have an immunosuppressive effect via the induction of antigen-specific IL-10–producing T cells [24, 26, 31]. With data emerging regarding the link between DC maturation and improved migration and the potential immunosuppressive role of the immature DCs and with the ability to infect either type of DC, we chose to concentrate our efforts on loading the mature DCs.

The distribution of Gag protein was mainly intracytosolic, as shown in figure 1*D*. The expression of Gag was detected very early and decreased over time, with a maximal expression at ~4–6 h after vaccine exposure. However, the ability to detect de novo synthesized protein (using a monoclonal antibody) within a DC is balanced by the fact that these cells are additionally processing and loading the antigen into MHC molecules. Along these same lines, the maturation level of the DCs may influence the rate and efficiency at which these protein products are processed (authors' unpublished results). Despite viral infection, protein production, processing and loading of MHC molecules, cytokine secretion, and cellular maturation, we found that the cell populations remained viable and were functional in assays of T cell stimulation.

Safety concerns currently require that all final products undergo stringent release testing prior to injection into humans. These tests, gel clot assays for the detection of endotoxin in particular, require at least 90 min to perform. Other logistics (e.g., cell phenotyping, viability testing, or volunteer arrival) may require that the cells remain in an injectable solution, as the tested and unaltered final product, for some time (several hours) prior to their injection. Maintaining vaccine loaded DC integrity in this injectable solution is an important goal of DC-based vaccinations. We found that keeping the DCs at 37°C, compared with 4°C, was best but that the cells clearly preferred medium or PBS to saline; however, no such buffered solutions exist that are suitable for injection. The best approach ultimately would be to inject antigen-loaded mature DCs as efficiently and safely as possible, allowing in vivo priming to commence.

We and others have reported previously that CP induces apoptosis in DCs [29, 30]. In these experiments, we found that higher MOIs (MOI, 10) and immature DCs resulted in a greater amount of DC apoptosis. Others have recently shown cross presentation of tumor antigen via the uptake of apoptotic DCs (induced by loading immature DCs with ALVAC encoding the melanoma-associated antigen, Melan-A/MART-1) in a melanoma model [30]. This latter observation raises the possibility that the apoptotic CP-loaded DCs could be cross-presented by DCs in vivo. We balanced the length of exposure to vaccine required for optimal loading and tolerable levels of apoptosis induced by vaccine exposure to allow a largely viable cell product.

Of interest, we did note a consistent increase in the ability of the CP-exposed DCs to stimulate T cells in both the mixed lymphocyte response (alloreactivity) and antigen-specific proliferation assays. This may be due to the associated maturation and activation effect from the CP itself. This suggests the potential for enhanced processing of endogenous proteins when a DC is exposed to appropriate maturational signals [43, 44], as has been shown in a murine model for exogenous MHC class II processing [45]. Finally, we found that the CP-loaded mature DCs could process and present relevant HIV antigens to autologous, IFN-γ–producing CD8 T cells (figure 5*C*). This has been demonstrated by others [46] and may support a role for DC immunotherapy in HIV-seropositive individuals, if the nature of the resultant CTL response could be improved. Indeed, the ability of DC vaccination to broaden the CTL response by expanding the T cell repertoire of a given epitope recently has been demonstrated using a transgenic mouse model and DCs loaded with a single peptide [47].

In summary, we have shown that GMP-grade lyophilized ALVAC-HIV vaccine constructs can be used to load clinical-grade human DCs with HIV genes. ALVAC-HIV vaccines induced TNF-α–mediated maturation of DCs. The vaccine-loaded DCs retained functional activity in allogeneic and autologous T cell assays. Vectored HIV genes were expressed early within the DCs. Of importance, expressed HIV proteins were processed and presented to sensitized $CD8^+$ T cells, resulting in HIV antigen–specific IFN-γ production in vitro. These results laid the foundation for an ongoing, proof-of-concept phase 1 trial, to optimize antigen presentation using ex vivo targeting of DCs in HIV-seronegative volunteers.

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